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Journal of Applied Ecology
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*Journal of Applied Ecology* 2014, **51,** 919–928 doi: 10.1111/1365-2664.12269

# Temperature- and latitude-specific individual growth rates shape the vulnerability of damselfly larvae to a widespread pesticide

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## Summary

**1.** Freshwater ecosystems are especially vulnerable to climate change and pollution. One key challenge for aquatic toxicology is to determine and manage the combined effects of temperature increase and contaminants across species' ranges.

**2.** We tested how thermal adaptation and life-history evolution along a natural temperature gradient influence the vulnerability of an aquatic insect to a pesticide under global warming. We applied a space-for-time substitution approach to study the effect of warming on the vulnerability of *Ischnura elegans* damselfly larvae to the pesticide chlorpyrifos in a common garden warming experiment (20 and 24 °C) with replicated populations from three latitudes spanning >1500 km in Europe.

**3.** Chlorpyrifos was more toxic to damselfly larvae at the higher temperature: mortality only occurred at 24 °C and the reductions in growth rate were stronger at 24 °C. This could partly be explained by parallel reductions in food intake but not by the activities of two widespread enzymatic biomarkers, glutathione S-transferase (GST) and acetylcholinesterase (AChE).

**4.** There was some evidence that the increased toxicity of the high chlorpyrifos concentration at 24 °C was stronger in terms of growth reduction in the faster-growing larvae from the low-latitude populations. This is consistent with energy allocation trade-offs between growth rate and pesticide tolerance, but suggests that local thermal adaptation does not play a role in coping with pesticide stress.

**5.** *Synthesis and applications*. Damselfly larvae from populations in lower latitudes were more vulnerable to a common pesticide at higher temperatures and pesticide concentrations, whereas evidence for the influence of local thermal adaptation on the vulnerability of larvae was weak. These results emphasize the need for spatially explicit bioassessment and conservation tools. Management practices aimed at mitigating pesticide run-off into aquatic ecosystems are particularly important in agricultural areas at low latitudes.

**Key-words:** chlorpyrifos, ecological risk assessment, global warming, latitudinal gradient, space-for-time substitution, thermal adaptation, voltinism

## Introduction

Freshwater biodiversity is particularly vulnerable to two major threats (Millennium Ecosystem Assessment 2005): global warming (Woodward, Perkins & Brown 2010) and contaminants (Bronmark & Hansson 2002). This is because most freshwater animals are ectothermic and unable to escape both stressors unless they emigrate. The ability of aquatic organisms to persist locally will therefore critically depend on their ability to deal with the combined effects of warming and contaminants, particularly pesticides (Noyes *et al.* 2009; Kattwinkel *et al.* 2011). This is a challenging topic for ecotoxicologists as temperature may interact with pesticides and modify their effects on natural populations (Noyes *et al.* 2009; Moe *et al.* 2013).

Considerable progress has been made in understanding how the vulnerability to pesticides changes at higher temperatures (Broomhall 2004; Noyes *et al.* 2009). Most studies are limited to populations occurring at a single

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latitude and therefore one local thermal regime. More information is required to generalize impacts across a species' range and provide an objective assessment of how local thermal adaptation may shape the effect of a temperature increase on the vulnerability to pesticides across the entire species range. Studies along natural thermal gradients such as latitudinal gradients may address this topic (Clements, Hickey & Kidd 2012). Assuming that other environmental factors do not differ systematically across latitudes, a careful choice of regions can provide an insight into how closely ecological responses to current temperatures in low-latitude regions will match predicted temperatures in 2100 in the high-latitude regions. In such a space-for-time substitution approach (Fukami & Wardle 2005), the vulnerability of individuals from a cooler region, at high latitudes, can be compared with the vulnerability of individuals from low latitudes that have adapted to cope with higher local temperatures. Despite its clear potential (De Frenne *et al*. 2013; Stoks, Geerts & De Meester 2014) and the surge of interest in studying the effects of contaminants on organisms under global warming (e.g. Noyes *et al*. 2009; Moe *et al*. 2013), this space-for-time substitution approach has been rarely applied in ecotoxicology (but see Dinh Van *et al*. 2013).

When relying on latitudinal gradients to study effects of thermal adaptation in relation to the vulnerability to contaminants, and generalizing toxicity tests across a species' range, it is important to consider latitudinal patterns in life history. Many species increase the number of generations per year at lower latitudes (Corbet, Suhling & Soendgerath 2006). This typically results in less time to grow and develop per generation and the evolution of higher individual growth rates, that is, higher increases in individual body mass during the larval stage, at lower latitudes (e.g. Ragland & Kingsolver 2007; Nygren, Bergstrom & Nylin 2008; Stoks, Swillen & De Block 2012). While the importance of population growth rates and the associated recovery rates across generations in shaping the vulnerability of populations to contaminants is well established (e.g. Barnthouse 2004; Liess & von der Ohe 2005; Kamo, Hayashi & Akita 2011), the potential effect of individual growth rates in shaping the vulnerability of individuals to contaminants during their life span has received less attention. This is, however, directly relevant for the standard toxicity tests that typically evaluate the vulnerability of a species to a contaminant based on individual effects (individual survival and individual growth rates) within a single generation (Walker *et al*. 2006). Life-history theory and, more specifically, energy allocation trade-offs predict that animals with higher individual growth rates will allocate more energy to growth and less to other functions, including defence (Sibly & Calow 1989; Congdon *et al*. 2001). We therefore hypothesize that animals from low-latitude populations with a faster life history should show a lower tolerance to contaminants compared to animals from high-latitude populations.

In this study, we tested for the role of thermal adaptation and life-history evolution along a latitudinal thermal gradient in shaping the vulnerability of an aquatic insect to a pesticide. We then used this to infer the vulnerability to the pesticide in the colder, high-latitude populations under global warming. We performed a common garden warming experiment, a standard method to assess local thermal adaptation in which animals from replicated populations of different regions are reared under a set of common temperatures. Specifically, we reared larvae from populations of low, central and high latitudes from the egg stage at two temperatures and under different levels of exposure to a pesticide in a factorial experiment. Larvae of the damselfly *Ischnura elegans* (Vander Linden) were selected as study organisms as damselflies are particularly vulnerable to global warming (Hassall & Thompson 2008). Comparisons of responses between the low- and high-latitude populations at their local temperatures should allow local thermal adaptation to be detected. The temperature difference between the low and high latitudes was selected to represent the predicted temperature increase by 2100 under IPCC scenario A1FI (IPCC 2007), allowing a space-for-time substitution approach to be applied to predict the vulnerability to the pesticide under global warming in the high-latitude populations. Finally, the faster life history at lower latitudes in this species (Shama *et al*. 2011; Stoks, Swillen & De Block 2012) allows the role of life-history evolution in shaping the vulnerability to a pesticide at the individual level to be assessed.

We tested the vulnerability to chlorpyrifos, an organophosphate insecticide, which is one of the most frequently used pesticides world-wide (Eaton *et al*. 2008). We documented how warming and pesticide exposure affect two key life-history traits in damselflies: survival and individual growth rate (Stoks & Cordoba-Aguilar 2012). To obtain mechanistic insights underlying the vulnerability of damselfly larvae to warming and pesticide exposure, we quantified the food intake and the activity of two widely used enzymatic biomarkers in freshwater invertebrates (Domingues *et al*. 2010): (i) glutathione S-transferase (GST), an important detoxification enzyme that is upregulated in damselfly larvae exposed to chlorpyrifos (Janssens & Stoks 2013a), and (ii) acetylcholinesterase (AChE), the target enzyme that is inhibited by chlorpyrifos (for damselfly larvae see Janssens & Stoks 2013a) and whose levels correlate with survival (Fulton & Key 2001) and individual growth rate (Janssens & Stoks 2013a).

## Materials and methods

### STUDY POPULATIONS AND REARING EXPERIMENT

We studied populations of the damselfly *I. elegans* from three latitudes spanning a north–south distance of *c*. 1500 km and comprising areas of the species' range distribution in low-latitude (southern France), central-latitude (Belgium) and high-latitude

(southern Sweden) areas in Europe (Gosden, Stoks & Svensson 2011). Gradual changes in voltinism, the number of generations per year, occur across these locations with three to four generations per year in southern France, two generations per year in Belgium and one generation every 2 years in Sweden (Corbet, Suhling & Soendgerath 2006). These voltinism changes are associated with a faster life history (growth and development) at low latitudes to compensate for the short growth period per generation. At each latitude, two randomly chosen populations from lentic shallow water bodies were sampled, namely Salette (+45°43′30·58″N, +5°22′23·92″E) and Arandon (+45°42′35·64N, +5°25′47·28″E) in France; De Maten (+50°56′39·89″N, +5°26′28·99″E) and Arenberg (+50°51′43·83″N, 4°40′53·96″E) in Belgium; and Kalmar Dämme (56°40′9·84″N, 16°17′48·48″E) and Långviken (+56°39′11·88″N, +16°20′2·76″E) in Sweden. These populations were chosen in natural areas without agriculture, and therefore, it is unlikely that they were exposed to pesticides (Coors *et al*. 2009). Furthermore, any local adaptation to pesticides would be unlikely in coenagrionid damselflies given their high levels of gene flow (Johansson *et al*. 2013).

In June 2010, we collected 10 mated females from each of the six populations and placed them individually in small plastic vials with wet filter paper for oviposition *in situ*. Eggs were transferred to the laboratory in Belgium. Throughout the experiment, eggs were incubated and larvae were reared at a water temperature of 20 °C or 24 °C and a photoperiod of 16(light):8(dark) hours. Temperatures of 20 and 24 °C reflect the mean summer water temperatures in ponds in southern Sweden and Southern France, respectively (see Appendix S1, Supporting information; De Block *et al*. 2013). Importantly, the 4 °C temperature difference also corresponds to the predicted temperature increase by 2100 under IPCC scenario A1FI (IPCC 2007). The crucial comparison to predict the vulnerability to the pesticide under global warming in the high-latitude populations is therefore the response to the pesticide of low-latitude larvae at 24 °C with the responses of the high-latitude larvae at 20 and 24 °C.

Ten days after hatching, larvae were allocated individually to plastic vials (7·5 cm height, 3·5 cm diameter) filled to a height of 6 cm with dechlorinated tap water. Vials were placed in temperature-controlled water baths and regularly redistributed among the three water baths per rearing temperature. All six water baths were placed in the same room to ensure equal rearing conditions (e.g. light regime). Larvae were fed *Artemia* nauplii *ad libitum* (459 ± 48 nauplii, mean ± SE, $n = 10$ food portions) 5 days per week. One day after moulting into the final instar, larvae were introduced in the exposure experiment at their respective temperature. Note that by doing so, all larvae had been acclimated to their experimental temperature (starting from the egg stage) before we tested effects of temperature and the pesticide.

## EXPERIMENTAL DESIGN

To test whether the effects of chlorpyrifos (CPF) exposure depend on temperature and latitude of origin, we set up a nested full factorial design with two populations per latitude × three latitudes × two temperatures (20 and 24 °C) × 3 CPF concentrations (0, 1·5, 3 μg CPF $L^{-1}$). Owing to some mortality during the pre-exposure period, the numbers of larvae exposed per combination of latitude × temperature × CPF concentration varied between 22 and 45 (total = 621 exposed larvae). Sample sizes per response variable are shown in the figures.

The CPF concentrations were chosen based on a range finding experiment where final instar larvae were exposed to a range of CPF concentrations (0, 1, 2, 3, 6 μg $L^{-1}$) for 6 days in a static renewal experiment with daily renewal of the medium. We chose the lowest CPF concentration where a growth reduction was detected compared to the control (=LOEC) being 3 μg $L^{-1}$ and half of this concentration (1·5 μg $L^{-1}$). These CPF concentrations are in general very high, but a 6-day exposure regime to these CPF concentrations is realistic in water bodies adjacent to agricultural lands where the study species can be very abundant (Appendix S2, Supporting information). A stock solution obtained by dissolving CPF powder (Sigma-Aldrich, purity >99%) in absolute ethanol was kept in amber glass bottles and stored in the dark at 4 °C. The CPF exposure solutions were daily prepared by diluting the stock solution with synthetic pond water (for details see Janssens & Stoks 2013b). The synthetic pond water was used as control; growth rates of damselfly larvae in this synthetic pond water do not differ from those observed in natural pond water (Janssens & Stoks 2013b).

At the start of the 6-day exposure period to CPF, final instar larvae were transferred individually to 100-mL glass jars. The sides of the jars were covered with brown tape to make sure that the larvae could not see each other (damselfly larvae are cannibalistic and impose predation threat upon each other, De Block & Stoks 2004). Each jar was filled with 50 mL of one of the three CPF solutions (0, 1·5, 3 μg $L^{-1}$), and the medium was refreshed daily (static renewal experiment). Another experiment in similar conditions showed that the CPF concentrations were still close (>85%) to the initial concentrations after 24 h (Janssens & Stoks 2013a). During the 6-day exposure period, the larvae were fed daily the same amount of *Artemia* as during the pre-exposure period.

## RESPONSE VARIABLES

We checked for mortality daily. To obtain an estimate of growth rate, each larva was weighed before (initial wet mass) and after (final wet mass) the 6-day exposure period to the nearest 0·01 mg using an electronic balance (AB135-S, Mettler Toledo®, Zaventem, Belgium). Each larva was gently blotted dry with tissue paper before weighing to ensure that no water remained on the larva. This gives reliable wet mass estimates that strongly correlate with dry mass (Stoks *et al*. 2005). Individual growth rates were calculated as $(\ln_{\text{final mass}} - \ln_{\text{initial mass}})$ 6 per days (Stoks, Swillen & De Block 2012).

On day six of the exposure period, we quantified the food intake following the protocol of Dinh Van *et al*. (2013). For logistic reasons, it was not possible to measure this trait in all larvae. Therefore, we restricted this measurement to all larvae from the low (France) and high (Sweden) latitudes. To quantify food intake, each larva was acclimated for 7 min in a plastic observation container (15 × 10 × 12·5 cm) filled with 600 mL of medium corresponding to the temperature and CPF treatment of a given larva. A separate test showed that the 7-min acclimation period was enough to provide reliable estimates of food intake that reflected the patterns of food intake during the exposure period in the exposure vials (see Appendix S3, Supporting information). *Artemia* nauplii (3560 ± 298 nauplii, mean ± SE, $n = 5$ food portions) were then added to the container, and the number of nauplii consumed in 7 min was monitored by direct observation. The swimming speed of *Artemia* nauplii, which determines

their vulnerability to damselfly larvae, was not modified after exposure to CPF for 7 min, neither was it temperature-dependent (see Appendix S4, Supporting information). This indicates that any differences in food intake among treatments is likely to reflect differences in foraging activity of the damselfly larvae. Food intake was expressed as the number of *Artemia* nauplii that a larva had eaten per mg larval wet mass per min (the average absolute food intake per larva and the average larval wet mass are given in Appendix S5, Supporting information).

At the end of the exposure period, all larvae were frozen at −80 °C for later spectrophotometric quantification of the activities of AChE and GST (for detailed protocols see Appendix S6, Supporting information). One unit GST activity represents the formation of 1 μmol GS-DNB per minute per mg protein. One unit AChE activity represents the hydrolysis of 1 μmol acetylcholine per minute per mg protein. The means of the three GST and AChE activities quantified per larva were used for statistical analyses. Twenty larvae (ten per population) from each of the 18 treatment combinations (3 latitude × 2 temperature × 3 CPF concentrations) were used for GST and AChE measurements (total of 360 larvae).

### STATISTICAL ANALYSES

We tested for effects of latitude, temperature and CPF on survival using a loglinear model. To test for effects of latitude, temperature and CPF on the response variables growth rate, food intake, GST activity and AChE activity, we ran separate ANOVAS using the mixed model procedure of SAS v9.3 (SAS Institute Inc., Cary, NC, USA). In all models, population nested in latitude was included as a random factor. As a stronger test for the hypothesis that the faster-growing larvae of the French populations are less tolerant to CPF, we specifically compared the effect of the high CPF concentration at 24 °C between the French and the Swedish populations.

Given that pesticide effects on growth rate may partly operate through changes in food intake, we tested for a covariation pattern between food intake and growth rate using an ANCOVA with latitude, temperature and CPF as independent variables and food intake as a continuous covariate. We initially included interactions with the covariate in the model, but as these were never significant, they were removed from the final model.

## Results

### LIFE HISTORY

Mortality did not differ across latitudes (Loglinear model, $\chi^2_2 = 1{\cdot}83$, $P = 0{\cdot}40$, Fig. 1). Survival was nearly 100% in all CPF-by-latitude treatment combinations at 20 °C and in the control treatment at 24 °C. However, at 24 °C, mortality occurred in CPF-exposed larvae with survival decreasing to ca. 80% in the high CPF concentration treatment (Temperature × CPF: $\chi^2_2 = 10{\cdot}09$, $P = 0{\cdot}0064$; Fig. 1b). This CPF effect at 24 °C did not differ among latitudes (Temperature × CPF × Latitude: $\chi^2_2 = 1{\cdot}03$, $P = 0{\cdot}90$), or when contrasting only the larvae from low latitudes (France) and high latitudes (Sweden) at high CPF ($\chi^2_1 = 0{\cdot}06$, $P = 0{\cdot}81$).

Overall, larvae from France had the highest growth rate (main effect Latitude, Table 1, Fig. 2). Growth rate was higher at 24 °C than at 20 °C in the control treatment, yet in the presence of CPF, the pattern reversed with growth rates being lower at 24 °C (Temperature × CPF, Table 1, Fig. 2). Overall, growth rates decreased with increasing CPF concentration. This growth reduction depended strongly on temperature and on latitude. Growth rates in the presence of CPF decreased stronger at 24 °C than at 20 °C (Temperature × CPF, Table 1, Fig. 2). The Latitude × CPF interaction indicated that while growth rates of larvae from Belgium and Sweden were already reduced at 1·5 μg $L^{-1}$ CPF (contrast analyses of control larvae vs. larvae at 1·5 μg $L^{-1}$; France: $P = 0{\cdot}63$, Belgium: $P < 0{\cdot}0001$, Sweden: $P = 0{\cdot}016$), growth rates of larvae from France only decreased at 3·0 μg $L^{-1}$ CPF (Table 1, Fig. 2). The specific test at 24 °C and the high CPF concentration showed a trend that under these conditions, larvae from French populations exhibited a stronger growth reduction (98%) than larvae from Sweden (49%) ($F_{1,118} = 2{\cdot}92$, $P = 0{\cdot}09$).

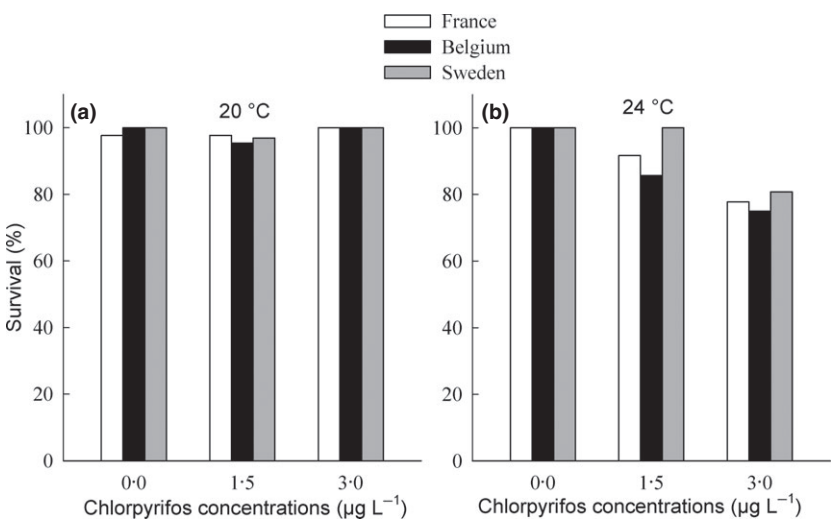


**Fig. 1.** Survival percentage of *Ischnura elegans* larvae in response to chlorpyrifos, temperature and latitude.

**Table 1.** The results of ANOVAS testing for the effects of latitude, temperature and chlorpyrifos concentration on growth rate, food intake, and activities of glutathione S-transferase (GST) and acetylcholinesterase (AChE) in *Ischnura elegans* larvae

| | Growth rate | | | Food intake | | | GST | | | AChE | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Effect | d.f.1, d.f.2 | *F* | *P* | d.f.1, d.f.2 | *F* | *P* | d.f.1, d.f.2 | *F* | *P* | d.f.1, d.f.2 | *F* | *P* |
| Latitude (Lat) | 2, 586 | 4·67 | 0·0097 | 1, 3·61 | <0·001 | 0·96 | 2, 5·85 | 0·11 | 0·90 | 2, 5·97 | 0·44 | 0·66 |
| Temperature (Temp) | 1, 586 | 4·39 | 0·037 | 1, 392 | 109·68 | <0·001 | 1, 357 | 58·08 | <0·001 | 1, 353 | 10·34 | 0·0014 |
| Chlorpyrifos (CPF) | 2, 586 | 18·62 | <0·001 | 2, 388 | 52·60 | <0·001 | 2, 354 | 0·46 | 0·63 | 2, 351 | 9·76 | <0·001 |
| Lat × Temp | 2, 586 | 0·22 | 0·81 | 1, 392 | 0·01 | 0·92 | 2, 357 | 8·12 | 0·0004 | 2, 353 | 2·55 | 0·08 |
| Lat × CPF | 4, 586 | 3·89 | 0·0040 | 2, 388 | 1·89 | 0·15 | 4, 354 | 0·97 | 0·42 | 4, 351 | 0·54 | 0·70 |
| Temp × CPF | 2, 586 | 8·82 | 0·0002 | 2, 388 | 11·02 | <0·001 | 2, 355 | 0·63 | 0·53 | 2, 351 | 0·75 | 0·61 |
| Lat × Temp × CPF | 4, 586 | 1·41 | 0·23 | 2, 388 | 0·61 | 0·54 | 4, 355 | 2·55 | 0·039 | 4, 351 | 0·18 | 0·32 |

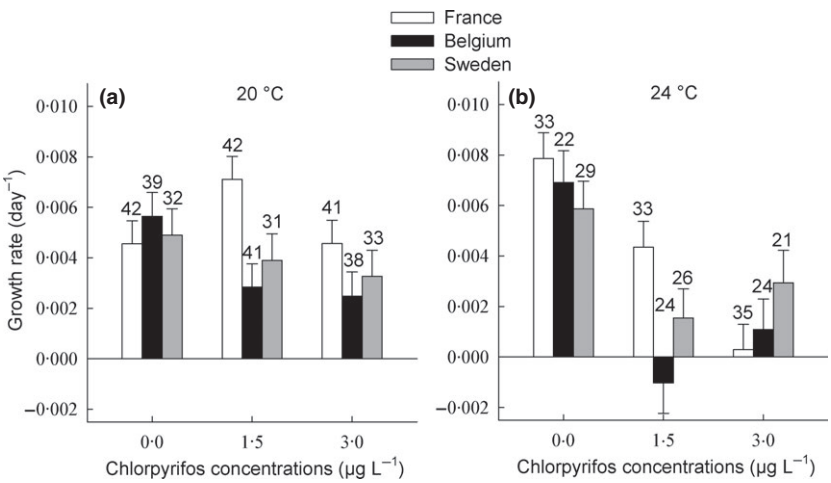


**Fig. 2.** Mean (+1 SE) growth rate of *Ischnura elegans* larvae in response to chlorpyrifos, temperature and latitude. Numbers above the bars represent sample sizes.

### FOOD INTAKE

Overall, larvae from France had a similar food intake compared to larvae from Sweden (Table 1, Fig. 3). Food intake was higher at 24 °C than at 20 °C (Table 1, Fig. 3). Food intake decreased with increasing CPF concentration (Table 1, Fig. 3). This decrease in food intake in CPF-exposed larvae was stronger at 24 °C than at 20 °C (Temperature × CPF, Table 1, Fig. 3); this interaction was similar across latitudes. Food intake positively covaried with growth rate ($F_{1,378}$ = 5·22, $P$ < 0·001, slope ± 1 SE = 0·0039 ± 0·0007).

### PHYSIOLOGY

Overall, there was no difference in GST activity among latitudes or CPF concentrations (Table 1, Fig. 4a,b). GST activity was higher at 24 °C than at 20 °C (main effect Temperature, Table 1, Fig. 4a,b). This temperature effect was more pronounced in larvae from Sweden (Latitude × Temperature), especially in the control and at 1·5 μg $L^{-1}$ (Latitude × Temperature × CPF, Table 1, Fig. 4a,b).

Overall, there was no difference in head AChE activity among latitudes (Table 1, Fig. 4c,d). AChE activity was higher at 24 °C than at 20 °C (main effect Temperature, Table 1, Fig. 4c,d). Exposure to CPF reduced AChE levels (main effect CPF, Table 1, Fig. 4c,d); this was similar across temperatures and latitudes (no significant interactions with CPF, Table 1).

## Discussion

Pesticides, including CPF (e.g. De Silva, Pathiratne & van Gestel 2009; Harwood, You & Lydy 2009), are generally more lethal to ectotherms at higher temperatures (reviewed in Noyes *et al*. 2009). In line with this, our study showed that exposure to CPF only resulted in increased mortality at 24 °C, but not at 20 °C. The uptake rates of CPF via the caudal gills have been shown to be faster at higher temperatures in aquatic insects (Buchwalter, Jenkins & Curtis 2003). The elimination rates are also thought to be faster at higher temperatures and it was concluded that the main reason for the higher toxicity of CPF at higher temperatures is the faster biotransformation into more toxic metabolites (Harwood, You & Lydy 2009).

Chlorpyrifos exposure had a negative effect on growth rate, which was also stronger at 24 °C compared to 20 °C. Growth reductions in pesticide-exposed animals have been observed in many aquatic taxa (e.g. insects: Tassou &

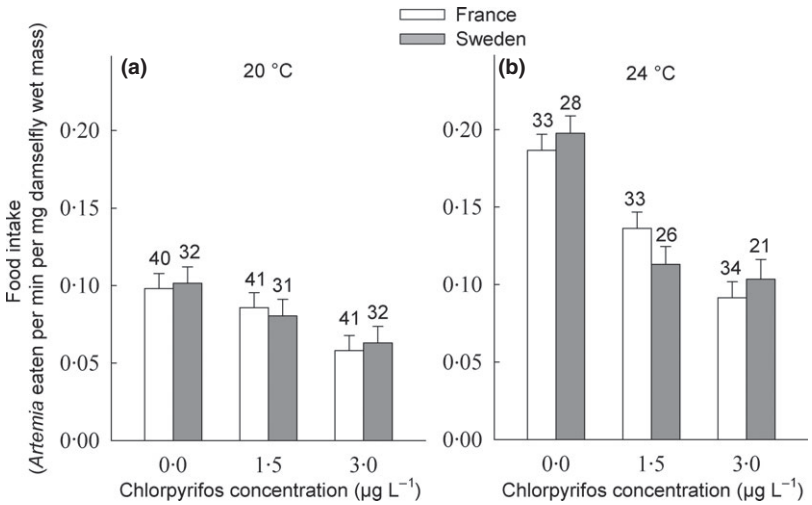


**Fig. 3.** Mean (+1 SE) food intake of *Ischnura elegans* larvae in response to chlorpyrifos, temperature and latitude. Numbers above the bars represent sample sizes.

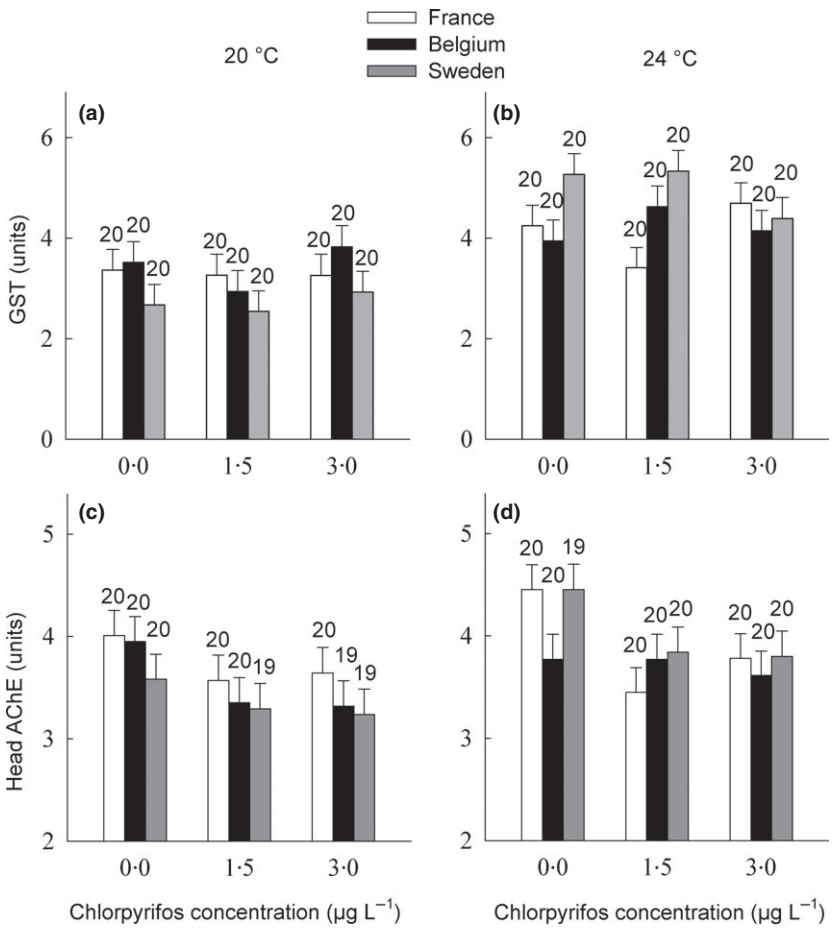


**Fig. 4.** Mean (+1 SE) glutathione S-transferase activity (a, b) and acetylcholinesterase activity levels (c, d) of *Ischnura elegans* larvae in response to chlorpyrifos, temperature and latitude. Numbers above the bars represent sample sizes.

Schulz 2012; amphibians: Broomhall 2004), including CPF-exposed damselfly larvae (Janssens & Stoks 2013a). In the present study, the growth reduction in CPF-exposed animals can be partly explained by an associated decrease in food intake. Indeed, food intake decreased in the presence of CPF and covaried positively with growth rate. This may have been further magnified by a reduced conversion of energy to biomass under pesticide exposure (Campero *et al.* 2007). Investment in other defence mechanisms against pesticide exposure, such as the upregulation of Hsp70 (Janssens & Stoks 2013a) and cytochrome P450 (Rakotondravelo *et al.* 2006), may also have contributed to the growth reduction in CPF-exposed larvae. The stronger growth reduction at 24 °C compared to 20 °C resembles the higher CPF-induced mortality at 24 °C and may be caused by the same mechanisms as discussed for mortality.

The reduced growth rates are likely to have negative fitness consequences such as delaying time to emergence and the lowering of emergence success (e.g. in imidacloprid-exposed midges: Pestana *et al.* 2009). Moreover, in agricultural areas with frequent spraying, it may increase the likelihood of exposure to multiple pesticide pulses (see Appendix S2, Supporting information).

We found no evidence for local thermal adaptation shaping the vulnerability of the damselfly larvae to CPF, instead there was some evidence for a higher vulnerability of the larvae from France at the high CPF concentration at 24 °C. This pattern is consistent with a resource-based allocation trade-off shaping the vulnerability at 24 °C (Sibly & Calow 1989; Congdon *et al.* 2001) whereby the faster-growing larvae from France allocate more energy to growth during the pre-exposure period and as a result are less tolerant during the exposure period. In line with this, levels of the stress protein Hsp70 are higher in larvae of *I. elegans* from Sweden than from France and show a negative covariation with growth rate (Stoks & De Block 2011). The lower investment in Hsp70 levels may be a proximate reason allowing larvae from France to grow faster than larvae from Sweden at the cost of a reduced tolerance to pesticides. The CPF-induced growth reduction at 1·5 μg CPF $L^{-1}$ was less strong in the faster-growing, multivoltine larvae from France. This is probably due to stronger selection to for faster growth rates in populations in France (to maintain multivoltinism) compared to larvae from higher latitudes, resulting in a better buffering of growth rates at low CPF concentrations, which was unlikely to be maintained at the high CPF concentration.

Despite the fact that GST and AChE are widely used biomarkers to assess pesticide contamination in freshwater invertebrates (Domingues *et al.* 2010), our results indicate that the activity of GST and AChE played no role in shaping the temperature-specific mortality pattern. Indeed, the GST activity was not affected by CPF, and while the AChE activity was moderately inhibited, this inhibition was similar at 24 °C and at 20 °C. The rather moderate inhibition of AChE may seem surprising at these high CPF levels, yet similar patterns have been reported in several other freshwater insects (Domingues *et al.* 2010). Furthermore, Buchwalter *et al.* (2004) showed that *in vitro* AChE activity in the homogenates of the midge *Chironomus riparius* was not inhibited by CPF even at much higher concentrations than the ones we used. One reason for this may be the rapid recovery of AChE levels or the increased activity of the enzyme carboxylesterase which provides alternative binding sites to the phosphoryl group that plays an active role in the AChE inhibition process by organophosphates (Jokanovic, Kosanovic & Maksimovic 1996). Whatever the reason for the lack of temperature-specific responsiveness of both enzymatic biomarkers to CPF, this indicates that it may be misleading to rely on these widely used biomarkers to judge temperature-dependent effects of chlorpyrifos.

## SYNTHESIS AND APPLICATIONS

Pollution and climate change are two major applied ecological research topics of high policy relevance (Sutherland *et al.* 2006). Two key findings of present study are directly relevant for regulatory policy related to both topics and address two challenges in ecological risk assessment: the incorporation of the impact of global warming in ecotoxicology (Moe *et al.* 2013) and the extrapolation of toxicity tests across natural gradients (Clements, Hickey & Kidd 2012; Rubach *et al.* 2012). Information on these topics is timely and can inform revisions or implementation of legislation in Europe to make toxicity testing more effective towards management and protection of freshwater biodiversity under global warming. This is hardly needed as a recent study showed that sublethal pesticide doses that current European legislation considers environmentally protective are causing losses in taxa up to 42% (Beketov *et al.* 2013), while under global warming, pesticide use is likely to increase at higher latitudes (Kattwinkel *et al.* 2011).

First, our results indicate that while warming makes CPF more toxic to damselfly larvae as evidenced by patterns in survival, growth rate and food intake, there was no detectable signal of local thermal adaptation shaping the effect of warming on the pesticide's toxicity. All else being equal (assuming, for example, no other environmental factors to differ systematically across latitudes), our results suggest that gradual thermal evolution will not counter the effect of the predicted 4 °C temperature increase by IPCC scenario A1FI (IPCC 2007) on the pesticide's toxicity in high-latitude populations. This finding makes it especially relevant to take into account the effects of warming by specifically including temperature in standard toxicity testing (Bednarska, Jevtić & Laskowski 2013) to achieve more ecologically realistic risk assessment of environmental pollutants. Furthermore, an understanding of temperature effects on the toxicity of pesticides in water bodies near agricultural areas, which may be important sites for biodiversity, provides managers with a realistic assessment of vulnerabilities of species to agricultural pesticides in different thermal regions. Explicit consideration of warming impacts provides opportunities to develop more specific management plans. For example, our results highlight that damselfly populations living in shallow ponds adjacent to agricultural areas that become warmer in summer may be more vulnerable to the effects of pesticide run-off which may guide management plans to increase the width of non-sprayed buffer zones (e.g. Rasmussen *et al.* 2011; Beketov *et al.* 2013; Christen & Dalgaard 2013) to minimize the risk of pesticide run-off into these thermally sensitive ponds. A complementary management action may be to provide vegetated ditches around these ponds (Stehle *et al.* 2011) that additionally favour the biodegradation of pesticides (Babut *et al.* 2013) and may increase shade to mitigate temperature increases (Sridhar *et al.* 2004).

Such management plans will be especially valuable in warmer regions at lower latitudes within a species' range given the absence of local thermal adaptation in shaping the vulnerability to pesticides of important intermediate predators in freshwater communities like damselfly larvae.

Secondly, as predicted by energy allocation trade-off theory, our data suggest a lower tolerance to a higher concentration of CPF in terms of individual growth rate in the faster-growing French populations that have more generations per year compared to the Belgian and Swedish populations. In general, intraspecific life-history evolution along a latitudinal gradient can shape the vulnerability to pesticides and may make it difficult to extrapolate toxicity tests obtained at a single site across latitudes and hence across the species' range, a long-standing challenge in ecological risk assessment (Van den Brink 2008; Clements, Hickey & Kidd 2012). While the conjecture that the geographical variation in toxicity can be predicted based on changes in voltinism needs more empirical testing, it may be a promising mechanism for a spatially explicit ecological risk assessment framework for the management and protection of freshwater biodiversity.

## Acknowledgements

We thank P. Lambret and V. Nilsson-Örtman for collecting eggs and the associate editor, David Angeler and three anonymous reviewers for their constructive comments. K.D.V. is a PhD fellow of VOSP and benefited an IRO Supplement, L.J. is a PhD fellow of IWT-Flanders and S.D. is a PhD fellow of the Research Foundation-Flanders (FWO). Financial support for this research came from grants of FWO, the KU Leuven Research Fund (Excellence Center Financing PF/2010/07) and the Interuniversity Attraction Pole (IAP) project Speedy.

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*Received 24 December 2013; accepted 11 April 2014*
*Handling Editor: David Angeler*

## Supporting Information

Additional Supporting Information may be found in the online version of this article.

**Appendix S1.** Mean summer water temperatures in the study ponds.

**Table S1.** Comparison of the measured mean summer water temperatures with the simulated mean summer water temperatures in one low-latitude and one high-latitude pond inhabited by *Ischnura elegans*.

**Table S2.** Simulated mean summer temperatures for the regions covering the set of two low-latitude and the set of two high-latitude ponds included in current study.

**Appendix S2.** Motivation chlorpyrifos exposure regime.

**Appendix S3.** Validation of the food intake estimates in the observation containers.

**Table S3.** The results of the repeated-measured ANOVA testing for the effects of chlorpyrifos and the quantification method (across 3 h using the filtering method in the exposure containers vs. across 7 min using direct observation in the observation containers) on food intake of *Ischnura elegans* larvae.

**Table S4.** The results of a repeated-measured ANOVA testing for the effects of chlorpyrifos concentration and transferring on food intake of *Ischnura elegans* larvae.

**Fig. S1.** Comparison of the mean (+1 SE) food intake (number of *Artemia* nauplii eaten) of *Ischnura elegans* larvae in the absence and presence of chlorpyrifos based on two different quantification methods: (i) across 3 h using the filtering method in the exposure containers (white bars) and (ii) across 7 min using direct observation in the observation containers (grey bars).

**Fig. S2.** Comparison of the mean (±SE) food intake of *Ischnura elegans* larvae in the observation containers in the absence and presence of chlorpyrifos as a function of the duration of the acclimation period.

**Appendix S4.** Effects of CPF and temperature on swimming speed of *Artemia* nauplii.

**Fig. S3.** Mean (±SE) swimming speed of *Artemia* nauplii as a function of chlorpyrifos and temperature. Sample sizes are shown above the bars.

**Appendix S5.** Absolute food intake and average individual wet mass of the damselfly larvae.

**Fig. S4.** Mean (±SE) food intake per individual larva (a & b) and mean (±SE) wet mass of *Ischnura elegans* larvae (c & d) during the food intake trials as function of the different combinations of chlorpyrifos concentration, temperature and latitude. Numbers above the bars represent sample sizes.

**Appendix S6.** Assays for the quantification of the activities of the enzymatic biomarkers.

**Fig. S5.** Mean (±SE) activity of acetylcholinesterase (AChE) as a function of chlorpyrifos concentration. The open circle represents the negative control (no AChE).